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LIQUID CHROMATOGRAPHIC DETERMINATION OF DIASTEREOMERIC GLUTATHIONE CONJUGATES AND FURTHER DERIVATIVES OF α-BROMOISOVALERYLUREA IN RAT BILE AND URINE BY ELECTROCHEMICALLY GENERATED BROMINE

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SUMMARY

To study the glutathione conjugation of α-bromoisovalerylurea in the rat in vivo, a reversed-phase liquid chromatographic assay of the thioether metabolites in bile and urine was developed. Since α-bromoisovalerylurea has a chiral centre, two diastereomeric glutathione conjugates (in bile) and two diastereomeric mercapturates (in urine) can be expected. The separation characteristics of these metabolites and the corresponding cysteine conjugates were investigated. Whereas all thioether metabolites could be separated in one run, optimal separation of the diastereomers required different mobile phases for the glutathione conjugates (in bile) and the mercapturates (in urine). The glutathione conjugates were analysed with the ion-pairing agent sodium decanesulphonate in the mobile phase, but the mercapturates were analysed without an ion-pair-forming agent. For detection, on-line generation of a constant bromine level (100%) was used; bromine-reactive compounds result in a decrease of the amperometric response from the 100% baseline. This technique could be used in continuous automated operation and required little clean-up of the sample. Thus, the diastereomeric glutathione conjugates and mercapturates were quantified in rat bile and urine samples, respectively, by direct injection of the (centrifuged and diluted) samples on the column. The limit of determination of the respective metabolites was 9 and 2.6 ng in bile and urine, respectively. Incubation mixtures of α-bromoisovalerylurea with a rat liver cytosolic fraction or with isolated rat hepatocytes were chromatographed after deproteinization with a double volume of methanol. The limit of determination of the diastereomeric glutathione conjugates in the deproteinized incubation samples was 2.0 ng.

INTRODUCTION

In the detoxification of many xenobiotics, glutathione (GSH) conjugation plays an important role. Most GSH conjugates are pharmacologically and toxicologically inactive and, in the rat for instance, are mainly excreted in the bile. Subse-

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quent metabolism, especially involving the gut, liver and kidney, usually results in excretion of mercapturic acids (N-acetylcysteine conjugates) or cysteine conjugates in urine [1]. The hypnotic drug α-bromoisovalerylurea (BIU; Fig. 1) is used as model substrate to study GSH conjugation in vivo and in vitro [2,3]. Like many substrates for the GSH transferases (such as epoxides and halogen-containing substrates [4–10]) BIU contains a chiral centre. Thus, conjugation of racemic BIU with (optically pure) endogenous GSH [(*S*)-γ-glutamyl-(*R*)-cysteinylglycine] can result in two diastereomeric GSH conjugates in bile (IU-S-G/I and II) and two diastereomeric mercapturates in urine (IU-S-MA/A and B) (Fig. 1 [11]). In order to develop a suitable analytical method for the study of the stereoselectivity of GSH conjugation, the separation characteristics of the diastereomeric thioether conjugates, including the cysteine conjugate (IU-S-Cys), were investigated.

Since most substrates for GSH transferases contain an aromatic group [4–7], reversed-phase liquid chromatography (RP-LC) with UV absorbance detection is a convenient method for most GSH conjugates. However, with substrates lacking chromophoric or fluorophoric properties, such as allyl isothiocyanate [12,13], BIU [2,14] and α-bromoisovaleric acid [3], this is not possible, and detection at 210 nm cannot be used owing to the poor sensitivity and the very high background of matrix components. Then, radiolabelled substrates can be used to detect the conjugates or, in some cases (after derivatization), gas chromatography of the cysteine conjugates and mercapturates is possible [14–16]. However, the polar and high-molecular-mass GSH conjugates can only be analysed conveniently with RP-LC. Therefore, an LC detection system based on the thioether moiety would seem to be a selective, universal method.

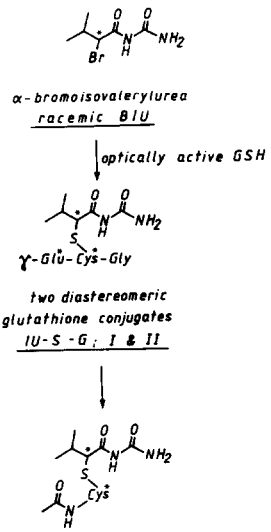

Fig. 1. Structure of α-bromoisovalerylurea (BIU) and its diastereomeric GSH conjugates (IU-S-G/I and II) and mercapturic acids (IU-S-MA/A and B). Chiral centres are indicated by asterisks.

A detection method for thioethers based on a ligand-exchange reaction has been described, but the sensitivity and selectivity were not satisfactory [17]. Therefore an indirect technique involving electrochemical detection [18–20] has been adapted. In this detection device, electrochemically generated bromine reacts with thioethers that are eluted from the LC column. Subsequently the excess of unreacted bromine is measured downstream. The amperometric signal reflects 100% bromine, and eluted compounds that react with bromine result in a decreased response. Bromine can be generated both off-line [18] and on-line [19,20]. In this study the latter method was applied and optimized to quantitate the diastereomeric thioether conjugates of BIU in bile and urine of rats after intravenous administration of the substrate. Furthermore, the diastereomeric GSH conjugates were analysed in incubation mixtures, such as isolated hepatocytes and rat liver subcellular fractions, to enable investigation of the stereoselectivity of GSH transferases in vitro.

EXPERIMENTAL

Chemicals

The cysteine and GSH conjugates of BIU and its mercapturates were synthesized as described previously [11]. Potassium bromide, citric acid monohydrate and LC-grade methanol were purchased from J.T. Baker (Deventer, The Netherlands). Sodium nitrate and sodium 1-dodecyl hydrogensulphate were obtained from Merck (Darmstadt, F.R.G.); sodium 1-decanesulphonate (DS) and sodium 1-octanesulphonate were obtained from Janssen (Beerse, Belgium). All solvents and reagents were of analytical-reagent grade and were used as such. Throughout the study deionized water was used (Milli Q purification system, Millipore, Bedford, MA, U.S.A.).

Chromatography

The chromatographic apparatus consisted of a Spectroflow Model 400 pump (Kratos Analytical, Ramsey, NJ, U.S.A.) equipped with a membrane-type liquid pulse damper, Model 12-0125, a WISP autoinjector (Millipore Waters, Bedford, MA, U.S.A.) injecting 5-μl samples and a Spherisorb ODS2 column (stainless steel, 15 cm$\times$3.0 mm I.D., packed with 5-μm particles). Isocratic elution was performed at a flow-rate of 0.45 ml/min. The dead volume of the system was 0.68 ml (1.5 min). The bromine generation cell was constructed at the department of Analytical Chemistry of the Free University (Amsterdam, The Netherlands). To generate constant amounts of bromine a laboratory-made galvanostat was used (current range 1–1000 μA; noise level $\leq$1%). Unless stated otherwise, the residence time in the reaction coil was 4.6 s (49 cm$\times$0.3 mm I.D.). The bromine level was measured with a Bruker LC/E230 potentiostat (Rheinstetten, F.R.G.) and a thin-layer transducer cell (BAS, West Lafayette, IN, U.S.A., Type TL-10A) with a platinum working electrode operating at +400 mV versus the BAS Type RE-3 reference electrode (Ag/AgCl/1 *M* LiCl in methanol–water, 1:1, v/v). At 5 μA generation current the detector current was of the order of 450 nA. With

TABLE I

SOLVENTS

Solvent No.	pH	Concentration of DS (m*M*)	Methanol (%, v/v)
1	2.5	0.1	—
2	2.5	—	—
3	2.5	0.1	20
4	2.5	—	15
5	3.5	5	25
6	Variable	—	20
7	3.5	—	Variable
8	3.5	Variable	20

fresh eluents the apparatus was conditioned overnight, and it was verified that equilibrium in chromatography and detection performance was obtained.

The eluent consisted of an aqueous buffer (0.1 *M* sodium nitrate as electrolyte, 0.01 *M* potassium bromide for the generation of bromine and 0.01 *M* citric acid as buffering agent and to chelate metal ions) and methanol in the presence or absence of sodium octanesulphonate, sodium decanesulphonate or sodium dodecylsulphate (Table I). The pH of the buffer was 2.5; to obtain higher pH values 4 *M* sodium hydroxide solution was added.

Sample pretreatment

Under sodium pentobarbital anaesthesia (60 mg/kg) the bile duct, urine bladder and external jugular vein of male Wistar rats were catheterized [21]. The rats had free access to food and water before the experiment. The BIU solution [50 μmol/kg; in a solution of 2% (w/v) bovine serum albumin containing 75 mg/ml D-mannitol; 5 ml/kg] was prepared by heating (80°C) and vigorous whirl-mixing, and was injected into the jugular vein. To ensure a high urine flow, a mannitol solution (75 mg/ml) was infused into the jugular vein at a flow-rate of 1.6 ml/h. At regular time intervals bile and urine samples were collected and stored in the dark at −20°C until the analysis took place [2,22]. Bile and (centrifuged) urine samples were diluted ten-fold with buffers 1 and 2, respectively (Table I). The bile samples contained only the diastereomeric GSH conjugates, and in the urine samples substantial amounts of only the diastereomeric mercapturates were found (see below [11]). Diluted bile and urine samples (5 μl) were injected on the column without further sample clean-up, using the mobile phases 3 and 4, respectively (Table I).

Incubation samples of BIU with rat liver subcellular fractions [22] or isolated rat hepatocytes [3], containing 1 m*M* GSH or methionine in the incubate, respectively, were deproteinated by addition of a double volume of ice-cold methanol and placing on ice. Samples were stored at −20°C in the dark and analysed by RP-LC after centrifugation (5 μl injected).

Variations in detector response were corrected for by injection of a standard

solution every 2–3 h. Solutes quantitation was based on peak-height measurements.

RESULTS AND DISCUSSION

Chromatography

The separation of the diastereomeric BIU metabolites was optimized by varying the pH of the mobile phase, the concentration of the modifier and the type and concentration of the ion-pairing reagent. In the absence of ion-pairing reagents (pH 2.5), the capacity factors (k') increased in the order IU-S-MA/A and B < IU-S-Cys < IU-S-G/I and II (Table II). The presence of ion-pair-forming agents increased the k' values of the metabolites with a (primary) amine function (IU-S-Cys and IU-S-G) but the k' value of the amide-containing mercapturic acid was not increased; in fact, the k' values of the IU-S-MA diastereomers even decreased (Table II). With octanesulphonate (5 m*M*; k' values between 1.5 and 2.0) the separation was less satisfactory than with DS (k' values between 1.0 and 3.1) or dodecylsulphate (k' values 0.4–3.3). The k' values of the IU-S-MA diastereomers in the presence of dodecylsulphate were low, and long equilibration times were required with this ion-pairing reagent, indicating that DS was the best choice.

The retention of IU-S-Cys and IU-S-G increased with increasing DS concentration, but the k' values of the mercapturates decreased as a function of the DS concentration (Fig. 2A). At DS concentrations greater than 5 m*M* no further change in retention was observed. This was not due to micelle formation since the critical micelle concentration of DS in eluent 3 was found to be 27 m*M*. Ad-

TABLE II

CAPACITY FACTORS OF α-BROMOISOVALERYLUREA CONJUGATES; INFLUENCE OF TYPE OF ION-PAIRING REAGENT, pH AND METHANOL CONCENTRATION

Compound	Ion-pairing reagent (5 m*M*)*			pH**			pH***			Percentage of methanol (v/v)§			
	C_8	C_{10}	C_{12}	2.5	3.5	4.5	2.5	3.5	4.5	10	20	30	40
IU-S-Cys	1.87	3.11	3.31	7.97	2.60	1.65	1.98	1.38	1.80	3.85	1.38	0.63	0.32
IU-S-G/I	1.62	2.10	1.32	8.68	2.25	0.53	3.74	1.86	1.50	7.29	1.86	0.66	0.27
IU-S-G/II	1.96	3.04	2.33	12.3	3.15	0.70	4.12	2.04	1.50	7.75	2.04	0.66	0.27
R_s	1.2	2.7	2.8	4.0	2.4	0.6	0.9	0.7	0.0	0.9	0.7	0.0	0.0
IU-S-MA/A	1.47	1.03	0.43	2.16	1.16	0.37	7.51	3.66	3.13	13.1	3.66	1.37	0.61
IU-S-MA/B	1.68	1.03	0.43	2.16	1.16	0.37	8.69	4.48	3.98	17.1	4.48	1.60	0.61
R_s	1.0	0.0	0.0	0.0	0.0	0.0	2.6	2.2	2.3	4.8	2.1	1.1	0.0

*At pH 3.5, with eluent 5.
**With eluent 5 and 5 m*M* DS.
***With eluent 6 and no DS.
§At pH 3.5, with eluent 7 and no DS.

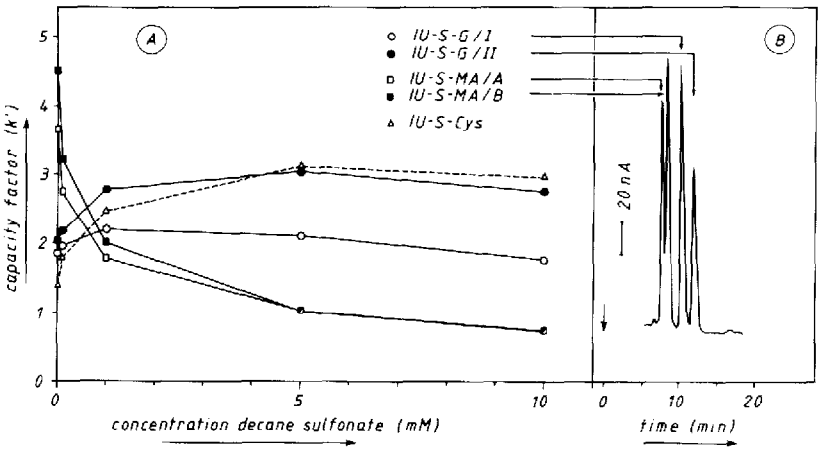

Fig. 2. (A) Capacity factors (k') of BIU metabolites as a function of DS concentration (eluent 8). (B) Separation of the IU-S-G diastereomers I and II and the IU-S-MA diastereomers A and B with eluent 3 (210, 177, 90 and 110 ng injected).

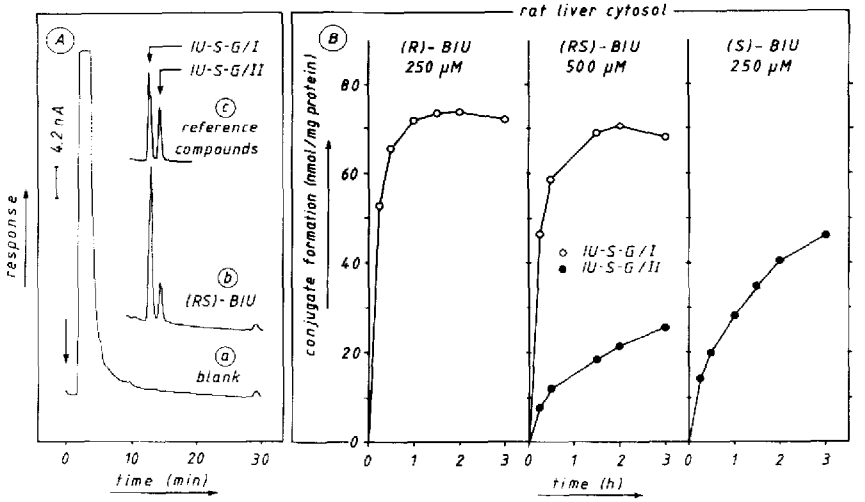

Fig. 3. (A) Chromatograms of deproteinated incubation samples of an incubation of 500 μM racemic BIU with a rat liver cytosolic fraction at (a) time zero and (b) after 3 h of incubation; (c) diastereomeric reference compounds IU-S-G/I and II (108 and 91 ng injected, eluent 3). (B) Representative example of the GSH conjugation of BIU by a rat liver cytosolic fraction. Incubations were performed with (R)- and (S)-enantiomers (250 μM) and the racemic mixture (500 μM total concentration) and contained 1 mM GSH and 4.2 mg cytosolic protein per ml.

equate separation of the IU-S-G diastereomers I and II was achieved at DS concentrations higher than 0.1 mM. In contrast, acceptable resolution of the IU-S-MA diastereomers was possible only at low DS concentrations (Fig. 2A). Under appropriate conditions, the separation of diastereomers IU-S-G/I and II and IU-S-MA/A and B was satisfactory in one run (eluent 3, Fig. 2B). Owing to matrix interferences in the urine samples, and since these samples contained only the diastereomeric mercapturates of BIU, these solutes were assayed in urine with

eluent 4 whereas IU-S-G containing bile samples were analysed with eluent 3. Similarly, incubation samples of BIU with rat liver subcellular fractions or isolated hepatocytes could be assayed with eluent 3 (Fig. 3A). Since the last eluting IU-S-G diastereomer had a retention time of ca. 12 min, ca. 96 samples could be analysed in 24 h. Owing to the presence of a biliary matrix component eluting at ca. 22 min, the throughput of bile samples with the same system was only 48 in 24 h. Also, 48 urine samples could be analysed in 24 h.

At increasing pH and concentration of the modifier, the k' values of all conjugates studied decreased (Table II), which is in accordance with the presence of two carboxylic acid groups in IU-S-G (the pK_a values of the two carboxylic groups in GSH are 2.1 and 3.5) and one carboxylic moiety in IU-S-MA (the estimated pK_a of N-acetylcysteine is 3.5–4). Elution of IU-S-Cys, however, was hardly influenced by the pH (Table II), in agreement with the low pK_a value of cysteine (1.7). Thus, IU-S-Cys is present as a zwitterion between pH 2.5 and 4.5. In the IU-S-Cys sample used, only one peak was observed with all eluents used, most likely because the synthesized product [11] contained only one of the IU-S-Cys diastereomers (unpublished results). In general, larger k' values due to less modifier or lower pH values resulted in a better resolution of the pairs of diastereomers (Table II). Ion-pairing reagents played an important role in the resolution of the diastereomeric GSH conjugates; at similar k' values of the GSH conjugates and mercapturates (k' ca. 4) a much better resolution of the diastereomeric GSH conjugates was obtained compared with the mercapturates ($R_s = 2.2$ and 0.9, respectively, Table II).

With acetonitrile instead of methanol, similar resolutions of the diastereomeric conjugates were found and no change in the retention of matrix components of bile and urine relative to the BIU metabolites was observed. Similarly, essentially the same chromatograms were obtained when a column with an I.D. of 3.9 mm instead of 3.0 mm was used (eluent flow-rates 0.8 and 0.45 ml/min, respectively).

Electrochemical detection system

As described by Kok et al. [20], thiols, thioethers and disulphides can be detected with the bromine-generation system. For an optimal performance many factors of the system have to be evaluated. The bromine-generation current (i_g) was verified to be linearly related to the 100% detector current (i_o) in the described system. Since the background noise is related to the absolute value of i_o, low generation currents have to be used to obtain low detection limits. An i_g of 2 μA was routinely possible and resulted in a noise signal of 40 pA ($i_o = 180$ nA, with a potentiostat time constant of 4 s). The background noise, mainly due to pressure variations, could be reduced to a half or a third by inserting a second pulse damper. To obtain linear calibration curves over a large concentration range, somewhat larger generation currents (5–20 μA) were required.

The selectivity of the detection system can be varied in several ways, such as by replacement of bromine by iodine, and changing the temperature or the length of the reaction coil. To improve the selectivity of the detection system for thiols in plasma, iodine instead of bromine was generated by Kok et al. [23]. We found that disulphides and thioethers were unreactive towards iodine, so that the io-

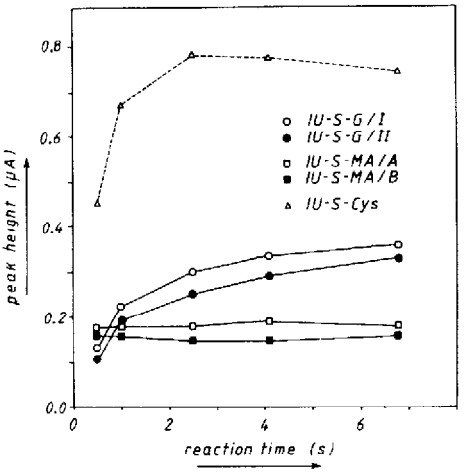

Fig. 4. Peak heights (per 2 nmol injected) of IU-S-Cys, IU-S-G/I and II and IU-S-MA/A and B as a function of the residence time in the reaction coil (19 cm of capillary corresponds to 1 s, 0.3 mm I.D.; LC column, 3.9 mm I.D.; flow-rate, 0.8 ml/min).

dine-generation system could not be applied. Variation of the residence time in the reaction coil showed that substantial differences in the rate and extent of oxidation by bromine existed between the BIU metabolites, although they all contain the same thioether moiety. The mercapturates reacted very quickly; less than 1 s reaction time resulted in the maximal response (Fig. 4). The cysteine conjugate reacted somewhat less rapidly (maximal response in ca. 3 s) and the oxidation of the glutathione conjugates was even slower (maximal response required more than 5 s, Fig. 4). Furthermore, different sensitivities were observed (different amounts of bromine were required to oxidize 1 mol of thioether). The sensitivity of the cysteine conjugate was higher than that of IU-S-G. The mercapturate was the least sensitive thioether metabolite. Finally, the sensitivity of the detection technique was influenced by the pH of the mobile phase and the concentrations of methanol and of the ion-pairing agent in the eluent. At lower pH values, with lower concentrations of the modifier and the ion-pairing reagent, an increased sensitivity was observed. In the presence of ion-pairing reagents (in particular with dodecylsulphate), long equilibration times (ca. 4–8 h) were needed to obtain a constant i_o.

The bromine-generation system was used satisfactory for 24 h a day in automated operation. The determination limit of GSH conjugates in incubation samples (determined as three times the background) was estimated as 0.9 μM (2.0 ng at $i_o = 100$ nA, eluent 3). At concentrations close to the detection limit (5–30 μM) linear calibration curves were obtained (correlation coeffient, $r = 0.997$, $i_o = 350$ nA). Determination limits of GSH conjugates and mercapturates in bile and urine, respectively, were 4.0 μM for IU-S-G (9 ng injected at $i_o = 300$ nA, eluent 3) and 1.8 μM for IU-S-MA (2.6 ng injected at $i_o = 350$ nA, eluent 4).

Quantitation of BIU metabolites and applications

Calibration curves of synthetic IU-S-G/I and II and IU-S-MA/A and B in water were linear over two decades ($r = 0.9990$; from 0.015 to 1.9 mM).

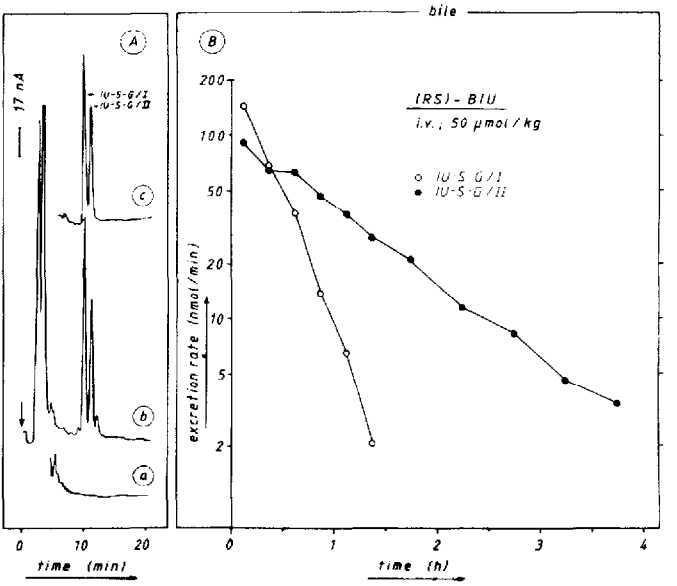

Fig. 5. (A) Chromatograms of (a) blank rat bile and (b) bile collected 15–30 min after intravenous administration of 50 µmol/kg racemic BIU to a rat; (c) diastereomeric reference compounds IU-S-G/I and II (178 and 132 ng injected, eluent 3). (B) Representative example of the excretion rates of glutathione conjugates in bile after intravenous administration of BIU (racemic mixture; 50 µmol/kg) to an anaesthetized rat.

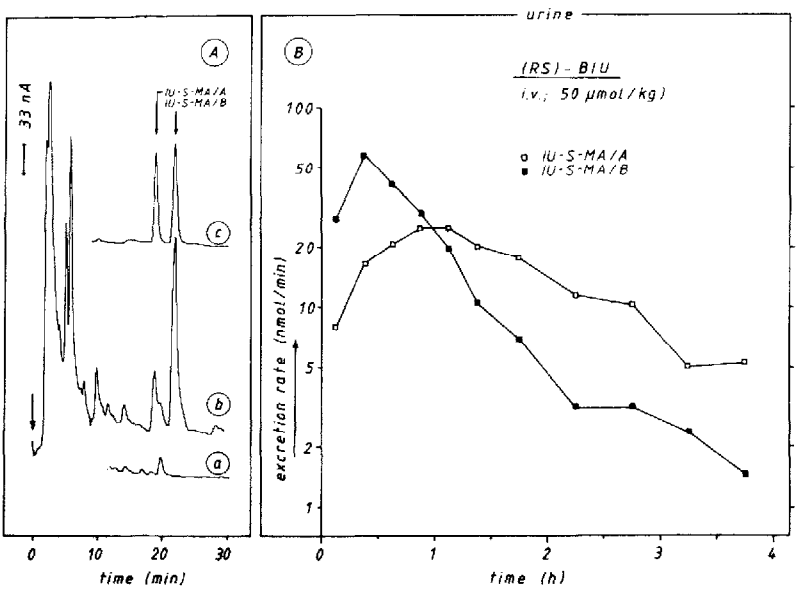

Fig. 6. (A) Chromatograms of (a) blank urine (15–30 min after start of the mannitol infusion [22] and (b) urine collected 30–45 min after intravenous BIU administration to a rat; (c) diastereomeric reference compounds IU-S-MA/A and B (467 and 562 ng injected, eluent 3). (B) Representative example of the excretion rates of BIU mercapturates in (mannitol-driven) urine from an anaesthetized rat after intravenous administration of racemic BIU (50 µmol/kg).

Simple sample pretreatment was sufficient to analyse the IU-S-G and IU-S-MA diastereomers. Recoveries from deproteinized incubation samples or centrifuged and diluted bile or urine samples was over 95%. In bile samples high concentrations of the diastereomeric GSH conjugates were found (0.015–2 m*M*), without interfering matrix components (Fig. 5A). Calibration curves showed good linearity over this concentration range. For IU-S-G/I: $y=0.252\ (\pm 0.029)x+0.48\ (\pm 1.35)$ ($r=0.998$, five samples per curve, mean of seven calibration curves); for IU-S-G/II: $y=0.201\ (\pm 0.021)x+0.31\ (\pm 0.48)$ ($r=0.9992$, $n=7$), where y and x are the peak height of the GSH conjugate peak (nA) and the amount of conjugate injected (ng), respectively. All seven calibration curves were prepared and analysed on different days; the number in parentheses reflects the day-to-day variation (S.D.). In urine samples, an interfering matrix component was present (Fig. 6A). The size of this peak decreased at later collection periods, similar to the decay in IU-S-MA concentration in the same samples, so that a satisfactory quantitation of the mercapturates was possible. Linear calibration curves of the mercapturic acids were obtained: for IU-S-MA/A, $y=0.217\ (\pm 0.040)x+0.04\ (\pm 0.69)$ ($r=0.9990$, five samples per curve, $n=7$); for IU-S-MA/B, $y=0.198\ (\pm 0.042)x+0.01\ (\pm 0.26)$ ($r=0.9995$, $n=7$).

The within-assay (within-day) precision was established as 1.6, 3.3, 0.7 and 1.8% for IU-S-G/I and II ($n=8$) and IU-S-MA/A and B ($n=5$), respectively (as S.E.M.: 42, 36, 23, and 28 ng injected, respectively). The samples were injected over a period of 24 h.

The chromatographic system could be used to study the stereoselectivity of GSH conjugation in vitro and in vivo; in incubations of BIU with a rat liver cytosolic fraction, a distinct difference in the GSH conjugation of the BIU enantiomers was observed: (*R*)-BIU, administered as pure enantiomer or racemic mixture, was conjugated more rapidly with GSH than the (*S*)-enantiomer (Fig. 3B). Similar results were obtained when BIU was administered to intact rats: the GSH conjugate of (*R*)-BIU (IU-S-G/I) was excreted in bile two- to three-fold faster than the conjugate of (*S*)-BIU (Fig. 5B). Furthermore, in urine the mercapturate of (*R*)-BIU was excreted faster than the diastereomer derived from (*S*)-BIU (Fig. 6B).

CONCLUSIONS

A determination of the diastereomeric thioether metabolites of BIU was developed; diastereomeric glutathione conjugates and mercapturic acids of BIU were quantified in bile and urine after intravenous administration of BIU to rats. The analysis enables investigations on the stereoselectivity of GSH conjugation in vivo. Furthermore, stereoselective GSH conjugation can also be studied in several in vitro systems since the formed diastereomeric GSH conjugates can be analysed conveniently. Continous automated operation was possible.

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REFERENCES

1 D.J. Reed and M.J. Meredith, in D.A. Roe and T.C. Campbell (Editors), Drugs and Pharmaceutical Sciences, Vol. 21, Drugs and Nutrients, the Interactive Effects, Marcel Dekker, New York, 1984, p. 179.
2 J.M. te Koppele, P. Dogterom, N.P.E. Vermeulen, D.K.F. Meijer, A. Van Der Gen and G.J. Mulder, J. Pharmacol. Exp. Ther., 239 (1986) 905.
3 J.M. te Koppele, I.A.M. De Lannoy, K.S. Pang and G.J. Mulder, J. Pharmacol. Exp. Ther., 243 (1987) 349.
4 L.A. Dostal, A. Aito, C. Harris, A.V. Bhatia, O. Hernandez and J.R. Bend, Drug Metab. Dispos., 14 (1986) 303.
5 C.C. Boehlert and R.N. Armstrong, Biochem. Biophys. Res. Commun., 121 (1984) 980.
6 J.B. Mangold and M.M. Abdel-Monem, Biochem. Biophys. Res. Commun., 96 (1980) 333.
7 B. Mannervik, Adv. Enzymol. Rel. Areas Mol. Biol., 57 (1985) 357.
8 J.B. Mangold and M.M. Abdel-Monem, J. Med. Chem., 26 (1983) 66.
9 D. Cobb, C. Boehlert, D. Lewis and R.N. Armstrong, Biochemistry, 22 (1983) 805.
10 I.G.C. Robertson, H. Jensson, B. Mannervik and B. Jernström, Carcinogens, 7 (1986) 295.
11 J.M. te Koppele, E.J. Van Der Mark, J.C. Olde Boerrigter, J. Brussee, A. Van Der Gen, J. Van Der Greef and G.J. Mulder, J. Pharmacol. Exp. Ther., 239 (1986) 898.
12 S.J. Borghoff and L.S. Birnbaum, Drug Metab. Dispos., 14 (1986) 417.
13 Y.M. Ioannou, L.T. Burka and H.B. Matthews, Toxicol. Appl. Pharmacol., 75 (1984) 173.
14 A. Niederwieser, B. Steinmann and A. Matasovic, J. Chromatogr., 147 (1978) 163.
15 N.P.E. Vermeulen, W. Onkenhout and P.J. Van Bladeren, in A. Frigerio (Editor), Chromatography and Mass Spectrometry in Biomedical Sciences, Elsevier, Amsterdam, 1983, p. 39.
16 W. Onkenhout, N.P.E. Vermeulen, W.C.M.M. Luijten and H.J. de Jong, Biomed. Mass Spectrom., 10 (1983) 614.
17 C.E. Werkhoven-Goewie, W.M.A. Niessen, U.A.Th. Brinkman and R.W. Frei, J. Chromatogr., 203 (1981) 165.
18 K. Isaksson, J. Lindquist and K. Lundström, J. Chromatogr., 324 (1985) 333.
19 W.Th. Kok, U.A.Th. Brinkman and R.W. Frei, Anal. Chim. Acta, 162 (1985) 19.
20 W.Th. Kok, J.J. Halvax, W.H. Voogt, U.A.Th. Brinkman and R.W. Frei, Anal. Chem., 57 (1985) 2580.
21 G.J. Mulder, E. Scholtens and D.K.F. Meijer, Methods Enzymol., 77 (1981) 21.
22 J.M. te Koppele, S.W. Esajas, J. Brussee, A. Van Der Gen and G.J. Mulder, Biochem. Pharmacol., 37 (1988) 29.
23 W.Th. Kok, J.J. Halvax, R.W. Frei, J. Chromatogr., 352 (1986) 27.